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VOLUME VI NUMBER 2

STAIN TECHNOLOGY

APRIL, 1931

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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention should be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is notknown, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME VI APRIL, 1931 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

How Much is Certification Worth?

Our attention has recently been called to the fact that a foreign stain company lists two grades of hematoxylin, one labelled "hematoxyline (dark)", the other "hematoxyline purest". In the catalogs of certain supply houses these grades are both listed, together with an American hematoxylin having no special designation other than the manufacturer's name. Inasmuch as this latter product sells in this country for somewhat less at present than the foreign "hematoxyline purest", the natural inference of the uninformed purchaser would be that it is not so highly purified. This raises the question how much actual value there is to the purchaser in the manufacturer's or the dealer's characterization of a stain by such terms as: "pure", "c.p.", "improved", etc. It is to be noted that such terms are used most often in the case of dyes such as hematoxylin whose actual purity cannot as yet be determined!

Now the American brand of hematoxylin above referred to is a well-known product advertised elsewhere in this journal with the designation "c.p." Altho absolute purity of a natural dye like hematoxylin is out of the question, we have never objected to the use of this designation on the part of the advertiser, because we regard this brand of hematoxylin as pure as any on the market. The actual dye content of hematoxylin, as above remarked, cannot be determined by methods yet devised; but this American brand seems to compare favorably with any foreign brand. The obvious conclusion is that in the case of this name the designation under which it is listed is of significance only in comparing with other grades from the same manufacturer.

It is apparent that those in the business of manufacturing or handling stains have a similar opinion as to the lack of significance of such designations. The manufacturers of the American hematoxylin write to us: "As far as our own hematoxylin is concerned, we attach little significance to the description 'C.P.' and feel that the word 'certified' is the one which has real meaning". We wonder if users as well as manufacturers agree in this.

What does certification mean? It means first that the manufacturer has produced the stain in coöperation with the Stain Commission, taking advantage of the latest information at our disposal as to the type of dye most satisfactory for the purposes for which this particular stain is used. Secondly, it means that a sample of the particular batch represented by the certification number on the label has been submitted to the Stain Commission and is kept permanently on file. It means, thirdly, that this sample has proved true to type, optically and chemically, and has a reasonably high dye content which is ordinarily specified on the label. In the fourth place, it shows that the sample has been tested in a biological laboratory under some member of the Stain Commission and has been found satisfactory for the purposes mentioned on the label. Lastly, it assures the purchaser that if the stain proves unsatisfactory, he can make his complaint to a disinterested body with experience to tell him whether his trouble arises from faulty technic or from a sample of stain that is not up to standard.

We have reason to believe that users of stains are already beginning to realize the value of this service. How much is it worth to them? Up to the present, the stain companies have made no extra charge for certified stains. The Stain Commission has made a small charge to the stain companies for certified labels, thus securing payment for a small part of the work involved in testing and certifying the stains; but the companies have absorbed this slight expense in their general scale of prices and have made no increase in the price of certified stains. In the future, however, they may find it necessary to charge some of the expense of certification to the consumer.

To explain the reason for this we must recall to the readers' attention the manner in which the work of the Biological Stain Commission has been financed. From the beginning, the chief means of support has been an appropriation from the Chemical Foundation whose generosity to this work and to other related activities is well known. It has always been realized that this method of financing the work could not be permanent; and if the work of the Stain Commission is to continue, some other source of support will eventually be necessary. The present industrial depression has affected the income of the Chemical Foundation and has naturally forced them to take careful account of their expenditures. As a result they have announced a small decrease in their appropriation to the Stain Commission for the year beginning with May 1, 1931. The present time, therefore, seems an appropriate occasion to look up other sources of income with the intention of making the work at least partially self-supporting.

The entire work of the Commission may be divided into three main lines: research; testing and certification of stains; publication of results. The publication work is self-supporting, and with the present subscription rate[1] of the journal will probably yield a small profit. The research work may possibly never pay its own way; but the testing and certification of stains really should be put on a paying basis.

The expenses chargeable to the testing and certification of stains amount to between $2000 and $3000 a year. If, therefore, this part of the work can be made self-supporting, the appropriation to be received from the Chemical Foundation can be devoted wholly to research, and the activities of the Commission in that line will not have to be curtailed because of the decrease in the appropriation. A plan has accordingly been worked out, proposed to the stain companies, and approved by them, whereby a larger income can be obtained from the services given in connection with certification. The companies are not anxious to pass this expense on to the customer, but to a certain extent they may have to do so. One of the chief objects of this notice is to explain the reason for any increase in the cost of certified stains that thus becomes necessary. The increase should not be large. An increase of about 5% in the average price per package of certified stain would yield the $2000 above named; and as the stain companies are already paying the Commission a quarter of that sum each year, the average increase to the consumer is likely to be under rather than over that figure. Surely the users of stains should be willing to contribute that small amount to the work of the Stain Commission.

H. J. CONN

[1]A slight increase has been made this year in the subscription rate of Stain Technology, in order to offset the decrease in our other funds. There is every reason, however, to believe that the amount of material published in the Journal will soon justify the increase; so that subscribers will continue to get their money's worth as well as feel that they are assisting in the support of the work.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

Stains Certified November 15, 1930 to March 1, 1931*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue	NA 5	87%	As histological and bacteriological stain	Nov. 25, 1930
Bismarck brown	NN 5	63%	As histological stain	Dec. 6, 1930
Phloxine	NPh 4	87%	As histological stain	Dec. 10, 1930
Brilliant cresyl blue	NV 10	...	As vital stain for blood	Jan. 6, 1931
Sudan IV	NZ 5	91%	As stain for fat	Jan. 7, 1931
Tetrachrome stain	NMn 5	...	As blood stain	Jan. 15, 1931
Methyl violet	NMv 2	81%	As histological and bacteriological stain	Feb. 15, 1931
Wright's stain	NWr 7	...	As blood stain	Feb. 15, 1931
Methyl orange	NM 3	87%	As histological counterstain	Feb 20, 1931
Methyl orange	CM 3	83%	As histological counterstain	Feb. 24, 1931
Basic fuchsin	CF 11	93%	For staining and for use in the Endo medium	Feb. 24, 1931
Eosin Y	CE 5	84%	As histological stain and as constituent of blood stains	Feb. 24, 1931
Crystal violet	CC 5	87%	As histological and bacteriological stain	Feb. 24, 1931

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

BLOCK STAINING OF NERVOUS TISSUE WITH SILVER

II. Trichloracetic Acid, Sulfosalicylic Acid, Hofker's and Carnoy's Fluids as Fixatives.[1]

H. A. DAVENPORT, *Institute of Neurology, Northwestern University Medical School, Chicago*

ABSTRACT.—**Alcoholic solutions of sulfosalicylic acid, trichloracetic acid and Hofker's solution fixed mammalian spinal cord with less shrinkage than ammoniated alcohol, but during the necessary alkalinization, washing, and silvering, the acid fixed specimens shrank more than those fixed in the alkaline alcohol. Specimens fixed in Carnoy's fluid shrank most. Successful silver stains were obtained after all the fixatives.**

The status of trichloracetic acid as a fixative was extensively reviewed by Hofker[2] in 1921. As a result of his experimental work, he recommended a mixture of 1 part trichloracetic, 1 part acetic acid and 8 parts of absolute alcohol as a general purpose fixative, and considered this mixture to be superior to aqueous or alcoholic solutions of trichloracetic acid alone. Sulfosalicylic acid was introduced in 1926 by Sannomiya[3]. He found that it could be used in both aqueous and alcoholic solutions and observed that shrinkage of tissue commonly seen in fixation with absolute alcohol was practically eliminated when it was added. Three per cent was sufficient concentration when used in absolute alcohol to prevent shrinkage.

Both of these protein precipitants have been used on nervous tissue, but so far as I have been able to find, not preliminary to silver staining. Cajal[4] used acetic acid in combination with formalin (Formula 8a) as a fixative, washed in water and then treated the tissue with ammoniated alcohol before transferring to the silver bath. Campbell's method[5] and Cowdry's[6] use Carnoy's fluid for fixation, the for-

[1]A previous article: "Studies of Fixatives, Lipid Solvents, and Reducing Solutions" appeared in STAIN TECHN., **5**, 139. 1930.

[2]Hofker, J. Die Trichloressigsäure als Fixierungsmittel. Zts. Wis. Mikr., **38**, 130. 1921.

[3]Sannomiya, N. Sulfosalizylsäure als Fixierungsmittel. Folia Anat. Jap., **4**, 363. 1926.

[4]Cajal, S. Ramon y. Las formulas del proceder del nitrato de plata reducido (y sus efectos sobre los factores integrantes de las neuronas). Univ. de Madrid, Lab. Biol., Trab., **8**, 1. 1910.

[5]Quoted from: Schneider, A. J., The histology of the radix mesencephalica N. trigemini in the dog. Anat. Rec., **38**, 321. 1928.

[6]Cowdry, E. V. The relation of mitochondria and other cytoplasmic constituents in spinal ganglion cells of the pigeon. Int. Monat. Anat. u. Physiol., **29**, 473. 1913.

mer employs ammoniated alcohol prior to silvering while the latter does not. De Castro[7] used a solution of nitric acid and chloral hydrate in 50% alcohol and later neutralized the excess acid with ammoniated alcohol. Three of these use ammoniated alcohol to neutralize excess acid before putting the tissue into the silver bath. Huber and Guild[8] fixed the tissue by injecting ammoniated alcohol thru an artery, decalcified with nitric acid, neutralized the acid with ammoniated alcohol and treated it with pyridine and a final washing in water.

These methods show that silver staining is feasible after acid fixation.

The questions involved in the present work are: 1) can the shrinkage which occurs after alkaline alcohol fixation be avoided by using acids (which are in themselves good protein precipitants) in alcoholic solution; and, 2) are satisfactory silver stains obtained after such fixatives? Ammoniated alcohol, unmodified absolute alcohol, and Carnoy's fluid were used as standards of comparison with the three other fixatives, because they represent established types of fixatives employed preparatory to silver staining.

The volume changes of blocks of spinal cord were followed during fixation and silvering as well as studies of the character of the stain seen after fixation in acid alcohol. Twenty-four blocks of spinal cord (12 cat and 12 dog) were fixed 20 hours in groups of 4 blocks (2 cat and 2 dog) in six different fixatives. The fixatives were: 1) absolute alcohol; 2) absolute alcohol containing 1% ammonia water; 3) 5% sulfosalicylic acid in alcohol; 4) 10% trichloracetic acid in alcohol; 5) 10% trichloracetic and 10% acetic acid in alcohol (Hofker's fixative); and 6) alcohol 70%, chloroform 30%, acetic 10% (Carnoy's fluid). The alcohol used in each of these fixatives was absolute. After fixation, all blocks were washed in one change of 95% alcohol for 3 hours and then two blocks of each series (1 cat and 1 dog) were transferred to 1% ammoniated alcohol and the remaining two to pyridin. The smaller blocks (cat) were left in the alkaline fluids 24 hours and the large (dog), 48 hours and both series washed in many changes of distilled water for 24 hours. Silver impregnation was made with aqueous 2% silver nitrate at 38°C for 4 days. All specimens were put into 80% alcohol for 5 minutes to remove the excess of silver nitrate from the surface and finally reduced 8 hours in a 5%

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[7]De Castro, F. Technique pour la coloration du système nerveux quand il est porvn de ses ètuis osseux. Univ. de Madrid, Lab. Biol., Trab., 23, 427. 1926.

[8]Huber, G. C. and Guild, S. R. Observations on the peripheral distribution of the nervous terminalis in mammalia. Anat. Rec., 7, 252. 1913.

aqueous solution of pyrogallic acid to which 0.25% sodium bisulfite had been added to prevent oxidation by air. The material was embedded in paraffin and prepared for microscopic examination.

The changes in volume of the blocks were computed from measurements made with a vernier caliper of the length, breadth, and thickness: (a) of the fresh tissue; (b) after fixation; (c) after alkalinization; (d) after washing; and (e) after the reduction of the silver. These volume changes were similar after alcohol and ammoniated alcohol fixation (Fig. 1, I) and after sulfosalicylic acid, trichloracetic acid and

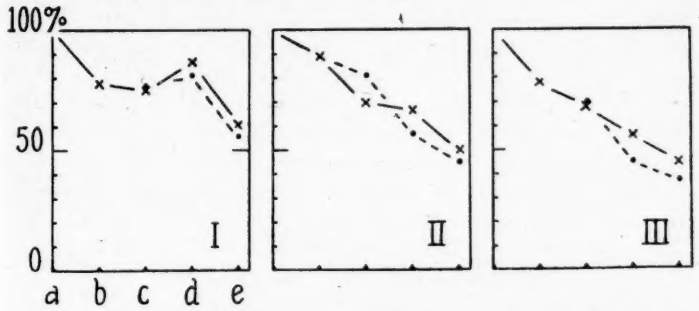

Fig. 1.—Charts indicating shrinkage of spinal cord during fixation and silver-staining. I. Averaged values obtained from fixation in 1% ammoniated alcohol and in unmodified absolute alcohol. II. The same for 10% trichloracetic acid, 5% sulfosalicylic acid, and Hofker's mixture. III. Carnoy's fluid.

a = original volume of unfixed tissue
b = volume after fixation
c = volume after ammoniated alcohol or pyridine
d = volume after washing
e = volume after silvering and reduction

The symbols x — x are used for the tissue treated with ammoniated alcohol after fixation; - - - for pyridin treatment after fixation.

Hofker's fixative (Fig. 1, II), hence the values obtained after the first two fixatives were averaged, likewise, 3, 4, and 5. Carnoy's fluid caused more shrinkage than the other fixatives and is given separately (Fig. 1, III). An unexpected feature in the volume changes was the shrinkage during washing (c to d) in distilled water after acid fixation with subsequent alkalinization. Measurements of the cross sections of the cut and mounted material indicated that the size of the blocks after silvering decreased uniformly about 10% during dehydration and embedding.

The results show that altho alcoholic solutions of trichloracetic and sulfosalicylic acids give a smaller initial shrinkage during fixation than ammoniated alcohol, the subsequent shrinkage is greater.

Reckoned on the basis of finished specimens, Carnoy's fluid caused the most shrinkage, while absolute alcohol and ammoniated alcohol caused the least.

With the exception of one block fixed in Carnoy's fluid, all specimens showed silver staining which might be considered successful and thereby indicated the feasibility of using either trichloracetic or sulfosalicylic acid in alcoholic solutions as a fixative. Aqueous solutions caused vacuoles to form in the tissue. The depth of penetration of the stain was less in the acid fixed specimens than in the others. However, there was less tendency to overstain at the periphery, hence spinal nerve roots and ganglia were well stained. Pyridin and ammoniated alcohol appeared to be equally efficient in neutralizing the excess acidity of the acid fixatives. Acid fixed specimens which were not alkalinized gave intense staining of cell nuclei and large fibers but small fibers were largely unstained and a granular precipitate was present in the interspaces between fibers. The function of alkalinizing nervous tissue appears to consist of bringing the isoelectric point of the protein[9] into a favorable zone for silver impregnation.

The observations of Hofker and Sannomiya as applied to fixation of nervous tissue were confirmed but the use of their fixatives does not prevent subsequent shrinkage during silver staining. Carnoy's fluid gave no special advantage in staining and caused the greatest shrinkage.

Data on shrinkage of spinal cord relative to fixation and silver staining should not be applied to other types of tissue. A few observations on embryos have indicated that under conditions similar to those applied here on adult spinal cord, the volume changes may be very different.

[9]A discussion of the work of J. Loeb relative to the combining power of gelatin and the silver ion is found on pp. 367 and 368 of Lee's Vade Mecum, 9th edition (1928).

A MODIFICATION OF THE TECHNIC OF HANDLING CHICK EMBRYOS[1]

F. B. ADAMSTONE, *Dept. of Zoology, Univ. of Illinois, Urbana, Ill.*

In the course of some work with chick embryos it was found necessary to devise some modification of the technic of handling them so that the process of fixation could be accomplished without delay and at the same time, retain the advantage of having the embryo spread out flat. The method ordinarily employed to obtain this last result is to remove the blastoderm from the yolk in normal saline solution, to float it into a Syracuse watch crystal and then, after removing the excess water, to spread it out upon a cover-slip. After this, the edges of the blastoderm are allowed to dry so as to attach it to the glass, but the embryo itself is prevented from drying by the addition of salt solution from time to time. About 10 to 20 minutes later, when the specimen is properly affixed, the killing fluid is added. The method has several drawbacks among which are: the difficulty of getting the blastoderm to stick satisfactorily, the danger of over-drying, the delay in fixation and the general loss of time. The following simple modifications were found so effective in eliminating the difficulties involved and are so easily introduced into laboratory practice, that they are offered for the consideration of other workers.

The embryo, with a generous excess of blastoderm is removed from the yolk and spread upon a cover-slip in the usual manner in saline solution, and, when the cover-slip is lifted, the edges of the blastoderm fold under. At this stage, instead of allowing the margin of the blastoderm to dry, the cover-slip is placed immediately upon a dry strip of paper in a clean container. The paper should be cut to fit and should be as wide as the cover-slip but a little longer so that the excess of paper can be used to lift the specimen and for any notations regarding it. After remaining on the paper for a few moments, the killing fluid may be added—at first a few drops directly upon the embryo and then, in about a minute, enough to cover the chick. In an hour the specimen can be moved to another container without danger of loosening. For small embryos, e.g., 24 to 36 hour stages, cover-slips should be cut to convenient size with a diamond pencil.

The advantages of the method are obvious. There is no delay in fixation, and, since no time is lost while waiting for the embryo to become attached to the cover-glass, the removal of a second specimen can be begun immediately. In addition, the handling of a large number of embryos in partitioned containers becomes much easier. The

[1]Contribution Number 398.

danger of having the specimen come loose from the glass is reduced to a minimum provided it is not handled until about an hour after the fixative is added.

The chief difficulty that has been encountered is the tendency of the embryo to stick to the glass. This can be easily overcome by giving the cover-slip a very thin coat of vaseline or paraffin. This procedure introduces other difficulties in the handling of very young embryos due to the effects of surface tension. These are easily overcome, if there is sufficient excess of blastoderm, by lifting the specimen vertically out of the saline solution (with the surface of the glass horizontal). However, the coating of grease renders the ultimate removal of the embryo from the glass very easy since the xylol or other clearing agent dissolves the wax out from under the embryo leaving it free from the cover-slip.

The explanation of the effectiveness of this method is probably to be found in the fact that when the blastoderm comes into contact with the paper some albumen soaks down into it and is coagulated when the fixative is added. Obviously it is best not to use a very good grade of paper, an absorbent type such as mimeograph paper being preferable.

In class work in the laboratory the method has been so uniformly successful, due to the saving of time effected and the superior results obtained by its use, that it is offered for the consideration of other instructors.

METHOD FOR STAINING BACTERIAL FLAGELLA

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ABSTRACT.—**The following method of staining bacterial flagella is recommended for use on smears made from suspensions of 10 to 16-hour agar slant cultures, incubated 30 minutes at 37°C before spreading on thoroly cleaned and flamed slides:**

1. Cover with fixative (100 cc. of ¼ sat. aqu. solution picric acid, with 5 g. tannic acid and 7.5 g. ferrous sulfate).

2. Wash with tap water, dry and cover with Fontana spirochaete stain; heat to steaming and allow to act for 1 to 2 minutes. Wash in tap water. The stain is prepared as follows: To 25 cc. 2% $AgNO_3$ add dilute ammonia till the precipitate which forms redissolves; then add more $AgNO_3$ till a faint turbidity results. A clear solution is useless.

Our object in studying the staining of bacterial flagella, was to develop a technic which should be relatively simple.

A number of chemicals possessing varying qualities were utilized in our preliminary studies; we attempted to produce the desired result both by successive use of the reagents, and by the use of a single mixture of various reagents. It became apparent that with the substances which we used (phenol, tannic acid, chromic acid, picric acid, bichloride of mercury, ferrous sulfate, ferric sulfate, ferric chloride, gentian violet, silver nitrate, ammonia), it was desirable to attempt to obtain two results in the processes of fixation and mordanting. In the first place, fixation or possibly precipitation of proteins was essential; a saturated solution of ammonium sulfate aided, but was not essential; tannic acid was effective, but when combined with, or preceded, or followed by other reagents, such as phenol, chromic or picric acid, the optimum effect appeared to be obtained. Secondly, it appeared to be necessary before satisfactory staining could be obtained, that the fixed flagella should be exposed to some metal; (our assumption was that some of the metal combined with the precipitated protein). For this purpose, iron salts appeared to give most satisfactory results especially ferrous sulfate. (Silver salts also gave somewhat less satisfactory results).

It was of course necessary to mix the various reagents of the fixative-mordant; and the quantitative relations of individual substances determined, to a considerable degree, the efficiency of the

result. In some mixtures too much precipitate was deposited on the surface of the slide; or in other cases, it appeared that the colloidal fixative-mordant was possibly too highly peptized, and little was deposited on the slide. (An optimum deposit, finely granular, accompanied satisfactory flagellar staining).

For the staining of the flagella after fixation and mordanting, we attempted to use anilin dyes; gentian violet, fuchsin, methylene blue, with or without admixture of phenol, alkali or anilin. At best, however, these gave only a faint staining of the flagella in certain cases. We used also various combinations of silver nitrate and tannic acid, but obtained frequently a heavy precipitate which masked the flagella. Eventually, we used the silver-nitrate-ammonia mixture, such as is used in the Fontana spirochaete stain.

The complete method which was found most satisfactory in our hands is as follows:

1. Clean slides—slides must be washed in soap and water, dipped in chromic acid, rinsed in distilled water, dipped in acid alcohol, and then thoroly flamed.
2. Make an emulsion of organisms in distilled water; the emulsion must be very thin (i. e. just perceptibly turbid) and the organisms should be taken from the water of condensation of a 10 to 16 hour agar slant culture.
3. Incubate the bacterial emulsion for 30 minutes at 37° C.
4. Place with a pipette a few drops of the bacterial emulsion on the clean slide, spread by tipping or rocking the slide and drain off excess fluid.
5. Dry in air and do not heat.
6. Cover with fixative (fresh), heat to steaming, then allow to act for two minutes. Fixative is prepared as follows:

   100 cc. one-fourth saturated (aqueous) solution of picric acid
   5 g. tannic acid
   7.5 g. ferrous sulfate

   NOTE: In case one is dealing with flagella very hard to stain, it may be well to repeat the heating. The fixative will retain its qualities for no more than 48 hours.
7. Wash with tap water and dry.
8. Cover slide with Fontana spirochaete stain, heat to steaming, then allow to act for 1 to 2 minutes. Wash in tap water and dry. Stain must be freshly prepared and is made as follows:

   (a) To 25 cc. of 2% $AgNO_3$, add dilute ammonia till the precipitate which forms, redissolves.
   (b) Then add more $AgNO_3$, till a faint turbidity results. A clear solution is useless.

The silver staining appeared to give a far sharper picture than any staining with anilin dyes.

It must, however, be recognized that in any attempt at flagella staining, the essential features after the cleanliness of the slide, are the condition of the organisms at the time the spread is made, and the method of making the spread on the slide (the handling of the bacteria and rapidity of drying). In our hands, only a certain percent of spreads made will show satisfactory results, and the percent increases with the experience of the worker.

FEULGEN'S REACTION APPLIED TO PROTOZOA AND SMALL WORMS, MOUNTED *IN TOTO* IN VENETIAN TURPENTINE

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ABSTRACT.—**Permanent mounts of certain protozoa and small worms are obtained as follows: kill suspensions of the organisms with Feulgen's fixative (6% $HgCl_2$ in 2% aqu. acetic acid) for 3 to 24 hours. After pipetting off the fixative, add successively: 70% iodized alcohol; ditto, 30 minutes later; 50%, then 35% alcohol; 2 baths distilled water; normal HCl. Transfer to cold water and heat to 60°C for 4 to 5 minutes or longer. Cool under running water; and wash in distilled water.**

Stain 1 to 3 hours in Feulgen's fuchsin sulfurous acid (1 g. of a suitable basic fuchsin, e. g. rosanilin hydrochloride, boiled in 200 cc. water, cooled, and allowed to stand 24 hours after adding 20 cc. normal HCl and 1 g. sodium bisulfite). Pass thru 3 baths of 200 cc. distilled water with 10 cc. normal HCl and 1 g. sodium bisulfite. Transfer to water and then to 35%, 70%, and 95% alcohols successively. Counterstain with fast green FCF, orange G or eosin Y in 95% alcohol. Pass thru two changes of absolute alcohol.

Transfer to 10% Venetian turpentine and place in a dessicator; mount after the turpentine has become concentrated.

If sections instead of total mounts are desired, the material should go from absolute alcohol, thru alcohol-xylol and xylol to paraffin (or preferably paraffin of M. P. 56°C with 3% bees-wax). The paraffin may be added to the material in the test tube, and cooled after the organisms have settled. Then break the tube, trim a block, and cut.

Feulgen's stain[1], also called the nucleal or chromatin reaction is based on a color change that takes place when aldehyde is added to a solution of basic fuchsin decolorized with sulfurous acid. This form of decolorized basic fuchsin has been known for some time by chemists as Schiff's reagent for aldehyde, and by bacteriologists as the essential ingredient of the Endo medium. In both of these applications, as well as in the Feulgen stain, the reaction is made evident to the eye by the restoration of the natural color of fuchsin.

[1]Feulgen, R. and Rossenbeck, H. Mikroskopisch-chemischer Nachweis einer Nucleinsäure von Typus der Thymonucleinsäure and auf die darauf beruhende elektive Färbung von Zellkernen in Mikroskopischen Präparaten. Zts. Physiol. Chem., **135**, 203–248. 1924.

Feulgen's explanation of his staining reaction is that aldehyde is produced when thymonucleic acids are partially hydrolyzed; and to emphasize the influence of aldehyde in the matter, he proposes the name "nucleal reaction". Since chromatin consists largely if not almost wholly of these acids, cytologists welcomed Feulgen's microchemical method for detecting them[2]. Thus far however, its application has been restricted to paraffin embedded material and to smears.

It seemed desirable to devise a method for applying this reaction to the protoplasts of small organisms, which would avoid shrinkage and be more delicate than the smear method. Venetian turpentine proved a good mounting medium for protozoa and small worms when stained *in toto* by the Feulgen method.

Permanent mounts of *Paramecium caudatum*, *Dileptus anser* and *Pristina* (family Naididae) showing clear chromatin reaction may be obtained by the following method. Suspensions with the organisms are transferred to test tubes. It is important to have heavy suspensions for otherwise there will be danger of diluting the fixative. The animals are killed with Feulgen's fluid (6% mercuric chloride in 2% aqueous acetic acid), and left therein for 3 to 24 hours. The tubes may be centrifuged in the case of protozoa to avoid the loss of the latter; this however is not recommended, because it tends to cause clumping, and by the action of gravity alone most of the material settles to the bottom within reasonable length of time. The fixative is then pipetted off to within a few millimeters, of the sediment, and 70% iodized alcohol is added. Iodine, to be sure, is not recommended in Krause's description of Feulgen's reaction, because of the danger of aldehyde formation. It was used, however, in the present work, because it did not influence the reaction with the material above mentioned, nor with rabbit's testes. The procedure is repeated in about 30 minutes for the same amount of time, 50% alcohol is next added for 30 minutes, then 35%, and the material is passed thru 2 baths of distilled water.

The organisms are now ready for hydrolysis. This is accomplished by pouring normal HCl into the test tubes, letting it act for a few minutes and transferring it to a beaker with cold water, which is heated to 60°C. The test tubes are left there for 4 to 5 minutes or longer, after which they are placed under running water to cool. A distilled water bath follows.

The material is then covered with Feulgen's fuchsin sulfurous

[2]For a more detailed discussion of its application see Krause's Enzyklopädie der Mikr. Techn. 3, 1729. 1927.

acid.[3] (To prepare boil 1 g. basic fuchsin in 200 cc. of water; cool to 50°C., filter, add 20 cc. of normal HCl, cool to 25% and add 1 g. sodium bisulfite; allow to stand 24 hours or until decolorized). Stain 1 to 3 hours; prolonged staining does not harm the reaction. The material is then passed thru three sulfite baths 200 cc. distilled water with 10 cc. HCl and 10 cc. of a 10% aqueous solution of sodium bisulfite). This procedure must not be slighted or the stain may diffuse and the clear chromatin reaction will be ruined. Transfer to water and then to 35%, 70% and 95% alcohol, successively. Fast green, orange G or eosin is added to the 95% alcohol as a counterstain. A mixture of orange G with eosin is especially recommended for total mounts of *Pristina*, for then the digestive tract is orange while the rest of the body is pink; they may be left for a considerable time in orange-G-eosin, altho protozoa have to be watched, for they seem to overstain easily in a strong solution of orange G. Two changes of absolute alcohol follow.

The organisms are transferred to 10% Venetian turpentine in 100% alcohol, placed in a dessicator and mounted after the Venetian turpentine has become concentrated. It is usually necessary to add more turpentine to the dilute solution before the process is completed. Slides thus obtained have not faded nor changed for 3 months.

If sections are desired the material is passed thru alcohol-xylol to pure xylol, and embedded in the usual manner in paraffin (M. P. 56°) to which about 3% bees-wax has been added. This takes place in the same test tube by immersing it into ice water, and afterwards breaking the glass. Lucas' method of embedding protozoa[4] may also be used. *Pristina* is embedded in the usual manner and may be oriented as desired

Sections are then cut, fixed and dried. Paraffin is dissolved with xylol and the slides are ready for mounting in gum damar. No difference of Feulgen's reaction was observed in comparing sections of organisms stained before and after embedding; and the first therefore is preferred because it saves time.

The procedure described above is recommended for cytologic investigations of small animals. By this method it is possible to obtain numerous individuals on one slide, with well defined chromatin, based on a microchemical reaction, and with unshrunken cytoplasm.

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[3]Not all basic fuchsins are satisfactory in this technic. A sample from Coleman and Bell (certification No. CF-10) was used in the present work. It is important to have a sample of fuchsin which decolorizes after standing no longer than two days. Practically identical results have been obtained with Grübler's fuchsin f. B. and with a sample of rosanilin hydrochloride obtained from the National Aniline and Chemical Co.

[4]Lucas, M. S. Method of concentrating and sectioning Protozoa. Science, 70, 482–3. 1929.

A PRELIMINARY REPORT ON A COMBINED GRAM-PAPPENHEIM STAIN FOR FORMALIN FIXED TISSUES

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During an investigation of new dyes conducted by one of us (S. A. S.) under the auspices of the Milbank Memorial Fund,[1] a method especially applicable to the identification of Neisserian organisms in stained films was devised. Attempts were then made to employ the method as a tissue stain and it was discovered that it combined the differential features of the Gram stain and the Unna-Pappenheim stain for plasma cells.

TECHNIC

The method which has given excellent results is as follows:[2] Paraffin sections of tissue fixed in 10% formalin are prepared as usual for staining.

1. Flush with crystal-violet-phosphate solution 3 to 5 minutes: (The buffer solution is made according to the method of Clark and Lubs.)

Crystal violet	1 g.
Phosphate buffer 6.6 to 7.0	10 cc.
Aq. dest.	90 cc.

2. Decant and flush with iodine solution.

Sublimed iodine	2 gs.
N/1 Na O H	10 cc.
Aq. dest	90 cc.

3. Decolorize with Merck's pure technical acetone 10 seconds or less. Decant quickly and avoid the slightest drying of the section.
4. Flush the section with counterstain 1 to 2 minutes (2 parts 2% aqueous over-ethylated methyl green[2], with 1 part ⅓% aqueous pyronin yellowish; allowed to stand several hours before using. Mix well before using).

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[1]Scudder, Sara A. Report to the Medical Committee, Milbank Memorial Fund, 325 East 38th Street, N. Y. C. 1930.

[2]The dyes were obtained from the National Aniline and Chemical Co. Inc. New York, N. Y. The methyl green is very similar to Grübler's iodine green; the pyronin is apparently identical with Grübler's pyronin G. These two dyes were specially prepared by the National Aniline and Chemical Company in coöperation with the Biological Stain Commission; the former is soon to be put on the market named Methyl green National, bearing the certif. No. N G 8.

5. Wash in tap water. Blot but do not allow to dry.
6. Immerse quickly in oil of origanum Cretic. Differentiate less than 1 minute. Blot.
7. Clear in two changes of bergamot oil.
8. Blot, mount in balsam.

Procedures 1 to 5 should be performed with precision and the slide should be kept moist. The time allowed for counterstain is important since over-exposure removes the stain from Gram-positive bacteria. Over-differentiation in oil of origanum Cretic removes the counterstain from Neisserian organisms. Oil of bergamot does not effect the stain even if the slides are immersed over night.

The special counterstain can be used with the Hucker ammonium oxalate solution in the technic which has been described by Lillie,[3] and renders better differentiation than picric acid and fuchsin counterstains recently advocated by Brown and Brenn.[4]

The results are as follows: Gram-positive bacteria are purple black; reversal of the Gram stain has not been noted. The majority of Gram-negative bacteria appear faded pink; however, a small influenzal bacillus which has frequently been encountered in exudate, stains deep magenta. Neissarian organisms are deep magenta. The capsule of *B. Friedlander* stains a transparent lavender. Polynuclear cells have dark blue-purple nuclei and very faint lavender cytoplasm. Small lymphocytes have homogeneous very deep clear blue nuclei; the cytoplasm is unstained or a very faint magenta violet to magenta. The nuclei of plasma cells are dark navy blue with cartwheel arrangement; the cytoplasm is very homogeneous deep magenta. The eosinophile nucleus is like that of a polynuclear, with granules fairly distinct, staining reddish and occasionally purplish. Endothelial cells have pale blue nuclei; the cytoplasm is light pinkish lavender. Giant cells have nuclei similar to those of the endothelial cells, with the cytoplasm like that of the plasma cell. The red blood cells are bronze to red. (Illustrations in color have been incorporated in a separate paper by one of us (S. A. S.), now in press.)

[3]Lillie, R. D. A Quick Method for Staining Gram-Positive Organisms in the Tissue. Arch. of Path., **5**, 828–834. 1928.

[4]Brown, J. H. and Brenn, L. A Method for the Differential Staining of Gram-Positive and Gram-Negative Bacteria in Tissue Sections. Bulletin Johns Hopkins Hospital, **48**, 69–74. 1931.

GRAM REACTION IN CRUSHED YEASTS

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ABSTRACT.—**Thick smears of yeasts are dried, crushed between slides or cover-slips, stained with anilin crystal violet 1½ minute, Lugol's iodine solution 2 minutes, decolorized in undiluted anilin oil, and counterstained with 1% aqueous safranin. Photomicrographs are given of crushed yeasts, thus stained, which show a Gram-positive outer layer and a Gram-negative inner substance.**

If thick smears of yeasts are dried, crushed gently between slides or cover-slips, and stained by the Gram method but decolorized with anilin, many forms can be seen which strongly suggest a Gram differ-

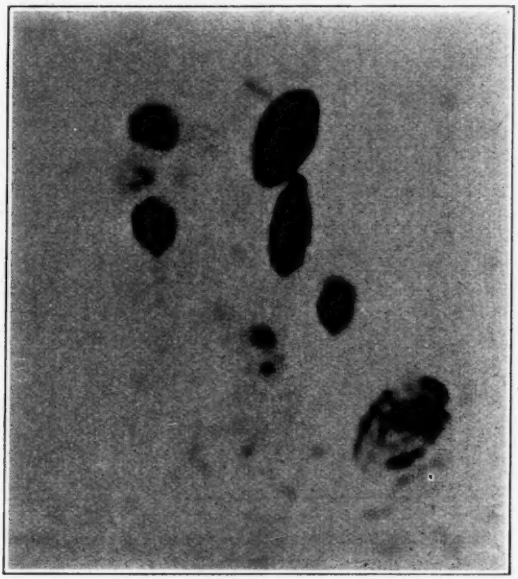

Figure 1. "Crushed *M. psilosis*, anilin-Gram stained 4400 diameters magnification. The rod shaped forms are the typhoid organism."

ence in the structure of the organisms, namely, a Gram-positive outer layer or cortex, and a Gram-negative inner or medullary substance. (See Kemp and Fleisher, 1926.)

In many trials with two strains of *Monilia* (*M. albicans* and *M. psilosis*) and an unidentified strain of *Monilia* together with several strains of commercial yeasts the results in the Gram staining of the

crushed forms were, very largely, those represented in the accompanying photomicrographs. The typhoid organism was used as a control for decolorization. It is necessary to state that prolonged and vigorous crushing will destroy all yeast forms in such smears with the result that all the stained material will be Gram-negative.

This bears some agreement with Churchman's publications (1927, 1929) on the mechanism of Gram staining in some organisms. His studies with *Bacillus anthracis* suggest, at least, an outer or cortical structure in this organism which stains Gram-positive, and an inner

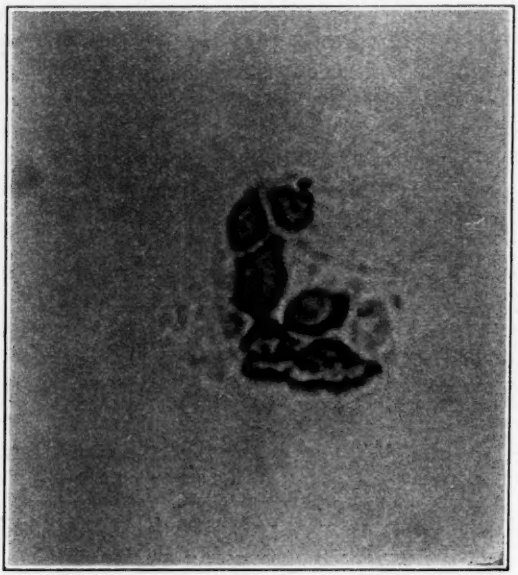

Figure 2. "Field "A:" Crushed *M. psilosis*, anilin-Gram stained 6200 diameters magnification."

or medullary substance which stains Gram-negative. The photomicrographs of smears of crushed yeasts made and stained as decribed above show what seems to be a similar Gram reacting structure in *Monilia albicans*, *M. psilosis*, and common yeast.

Burke and Barnes (1929) prepared smears of crushed yeasts in the same way but using the Burke (1922) method of decolorizing found that all crushed yeasts were Gram-negative. They reported: "the cell wall (yeast) does not stain with the dyes used in the Gram stain", defining the cell wall as the "sac" and "not the outer layer of proto-

plasm". Since Benians (1912) reported Gram-negative reactions in all crushed Gram-positive bacteria there has been a large number of publications substantiating his work. The report of Burke and Barnes is to be included among them. None of the published work so far observed describes the use of anilin as a decolorizing agent in such experiments. At the time of developing the anilin technic it was thought that anilin might serve better as a decolorizer in that it should not interfere so actively as alcohol, acetone, ether, etc., with the relatively unstable chemical compound assumed to exist, namely,

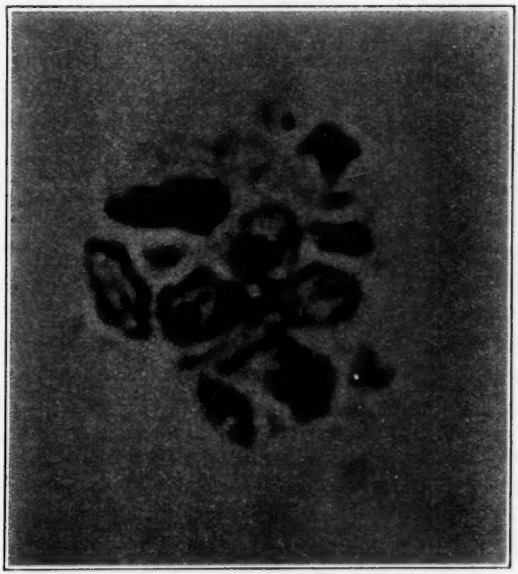

Figure 3. "Field "B," crushed *M. psilosis*, anilin-Gram stained 6200 diameters magnification."

"Gram-positiveness". The past four years of routine work, teaching experiences with medical and dental students, and experimental work such as this has strengthened this idea regarding anilin in the Gram stains. In smears of other crushed Gram-positive organisms the fragments were found to be too small to support even a supposition that they represented cortical structures of the original organisms. I believe, however, that the three Gram-positive organisms reported herewith do have an outer layer or cortex which shows Gram-positive staining.

The use of anilin as a decolorizer (Kemp & Fleisher, 1926) is not suggested as an improvement over the very efficient methods of Burke (1922) and others. The anilin method is more time-consuming, in that the slide must be dried before dipping in the anilin and dried after decolorizing in preparation for the aqueous counterstain. This drying is accomplished by blotting and careful flaming.

The staining technic followed in this work employed Stirling's gentian violet made up as follows:

Gentian violet (C. I. No. 681, i. e. crystal violet; Certif. No. NC-8; dye content 91%)	5 g.
Anilin	2 cc.
Ethyl alcohol, 95%	10 cc.
Distilled water	88 cc.

The anilin is first shaken up with the alcohol, then mixed with the water and added to the dye while grinding the latter in a mortar.

This staining fluid is applied for a minute and a half, followed by Gram's iodine solution (Lugol's) two minutes, decolorized in full strength anilin oil, C. P., and counterstained with 1% aqueous safranin (sample employed bore Certif. No. NS–7, with a dye content of 94%).

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NON-TOXIC DYES AND DYE-RESISTANT BACTERIA

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ABSTRACT.—**Exposure of three typical organisms—*Serratia marcescens* Bizio (syn. *Bacillus prodigiosus*), *Bacillus anthracis* Koch and *Staphylococcus aureus* Rosenbach—to saturated aqueous solution of neutral red (5.6% National Aniline certified) for 48 hours failed to kill them; and *Serratia marcescens* survived 9 days exposure. All three organisms survived 21 days exposure to 2% solution of neutral red and *Bacillus anthracis* survived thirty days exposure to this dilution of dye. The three organisms survived 24 hours exposure to saturated aqueous Bismarck brown (Grübler) and *Bacillus anthracis* survived 40 days exposure. *Serratia marcescens* (known to be one of the Gram-negative group resistant to triphenylmethane dyes) was tested for degree of resistance to gentian violet. The bacteriostatic titre of this dye in agar for this organism is about 1: 900. It was found to survive 4 hours exposure to 1: 2000 solution and is probably unaffected by even much stronger dilutions. To aqueous suspensions of this organism enough gentian violet was added to make a 1: 15,000 solution. Plants made from this specimen at the end of 26½ months exposure showed vigorous growth. In stronger solutions (1 drop of 1% dye to 3 cc. bacterial suspension) transplants made at the end of 16 months showed vigorous growth. With acid fuchsin even more striking results were obtained. Transplants from tubes containing 2 cc. of bacterial suspension of *Serratia marcescens* and 4 drops of 2% aqueous acid fuchsin (National Aniline) showed vigorous growth after 46 months exposure to the dye. No evidence of spore formation by *Serratia marcescens* was obtained. Bacteriocidal action of both gentian violet and acid fuchsin is greatly increased by slight rise of temperature.**

When, in the early days of anilin dyes, the capacity of certain of them to kill bacteria had been demonstrated, the general notion got about that all dyes are poisonous for all bacteria. As recently as 1902 L. Michaelis[2] wrote: "a strong solution of dyestuff is *eo ipso* sterile, for all dyestuffs are in high concentrations poisons for protoplasm and bacteria." This doubtless epitomized the ideas then held; and many still hold them.

[1]Work financially aided by the Chemical Foundation, Inc.

[2]Michaelis, L: Einführung in die Farbstoffchimie für Histologen, Berlin 1902. pp. 96.

The work of the last eighteen or twenty years on the selective nature of the behavior of dyes toward bacteria has indicated that these ideas of dye toxicity and bacterial resistance must undergo considerable modification to accord with the facts now at hand. In a later edition of the work of Michaelis, if one is ever published, the sentence quoted will no doubt be altered; for the author states in a letter that he regards the 1902 edition as "rather obsolete indeed."

In the present paper I wish to report a few observations which indicate the relatively innocuous nature of some dyes for many typical varieties of bacterial life and the degree of resistance of certain bacterial species toward dyes which are known to be highly toxic for many other species.

Non-Toxicity of Certain Dyes for Bacteria

Neutral red (National Aniline Co., Cert. No. NX 3, dye content 70%) and Bismark brown (Grübler; and National Aniline Co., Cert. No. NN4, dye content 42%) were the dyes used in the experiments dealing with non-toxicity. One-half cubic centimeter of heavy aqueous suspensions of 24-hour cultures of *Staphylococcus aureus* Rosenbach, *Serratia marcesens*, B1210, (syn. *B. prodigiosus*) and *B. anthracis* Koch[3] were put into three tubes each containing 1 cc. of saturated aqueous solution of neutral red (5.6%). The tubes were allowed to stand for 48 hours at room temperature, when transplants were made on to agar. All three organisms grew as vigorously as if no dye had been present in the tubes. The picture was the same as shown in Fig. 1. The bacteria, whether examined in fixed smears or in hanging drop, were lightly but definitely stained, altho in such cases it is impossible to be sure whether it is only the dead individuals in the specimen which have taken the stain.

After 96 hours exposure to the dye, *Serratia* still grew vigorously on transplant, but *Staphylococcus aureus* no longer grew, and *B. anthracis* grew only weakly. After 9 days' exposure to the saturated solution of the stain, both the *Staphylococcus* and *B. anthracis* failed to grow; but *Serratia* still grew vigorously when transplanted.

If, instead of a saturated solution of neutral red, a 2% solution be used, all three organisms will grow vigorously on transplant even after 21 days exposure (Fig. 1). After 35 days' exposure, *Staphylococcus aureus* is no longer alive; but *B. anthracis* and *Serratia* are still unaffected by their long stay in the dye. (Fig. 4, 2).

[3]The sources of these cultures were as follows: *Staphylococcus* isolated by Torrey from human feces, typical in every respect; *B. anthracis* from Amer. Type Culture Collection, No. 10; *Serratia* from Americam Museum of Natural History (before its cultures were taken over by the Society of American Bacteriologists and became the American Type Culture Collection), typical in every respect.

With Bismarck brown (Grübler), comparable tho not identical results have been obtained. If the same three organisms be exposed to a saturated solution of this dye (1.2%) all three are found to be alive and vigorous when transplanted at the end of 24 hours (Fig. 2). Yet two of these organisms (*Staphylococcus aureus* and *B. anthracis*) are known to be highly suceptible to the bacteriostatic action of the triphenyl methane dyes like gentian violet, and *Staphylococcus* is killed by 45 minutes exposure to 1: 4,500 solution of this dye. (Fig. 5, 1). *B. anthracis* put into saturated Grübler Bismarck brown 40 days ago, still grows vigorously on transplant (Fig. 3).

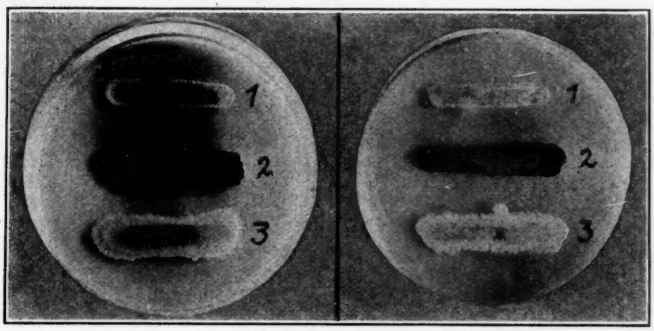

Fig. 1. Uninhibited growth of three organisms which have been planted after 21 days exposure to 2% neutral red.
1. *Staphylococcus aureus*
2. *Serratia marcescens* (Syn. *B. prodigiosus.*)
3. *Bacillus anthracis*

(The same picture was presented when transplants of these organisms were made after 48 hours exposure to saturated solution, i.e.5.6%.)

Fig. 2. Uninhibited growth of three organisms which have been planted after 24 hours exposure to saturated Bismarck brown (1.3%).
1. *Staphylococcus aureus*
2. *Serratia marcescens*
3. *Bacillus anthracis*

Resistance of B. Prodigiosus to Toxic Anilin Dyes

That Gram-negative bacilli are as a class resistant to the bacteriostatic and bacteriocidal effects of triphenyl methane dyes is now well known. Few are more resistant than *Serratia;* but it is perhaps not generally appreciated how great this resistance is. The experiments here recorded indicate the somewhat unexpected degree of its ability to survive exposure to gentian violet and to grow in its presence.

First, as regards resistance to the *bacteriostatic* properties of gentian violet. Determinations show that growth of *Serratia* on agar is pre-

vented by gentian violet only when the dye is used in dilutions of about 1: 900. How great such a resistance as this really is, may be appreciated by comparing it with the bacteriostatic titre of gentian violet for *B. anthracis*, whose growth on agar is prevented by dilutions of 1: 650,000 and, in broth by very much weaker dilutions.

Serratia marcescens is also relatively resistant to the *bacteriocidal* effects of this dye. For example, if *Staphylococcus aureus* be exposed for 45 minutes to 1: 4,500 solution it is killed or greatly damaged (Fig. 5, 1) while the *Serratia* readily survives 4 hours exposure to 1:

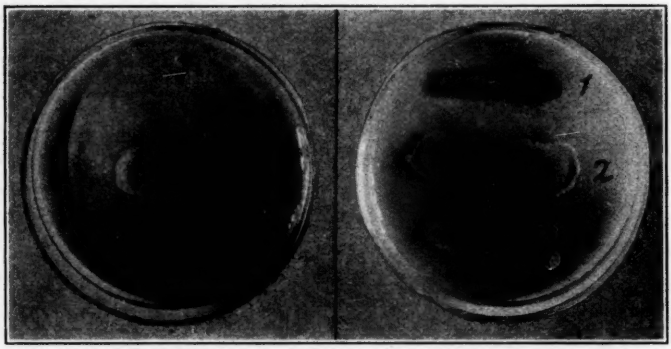

Fig. 3. Streak inoculation of *B. anthracis* after 40 days exposure to Bismarck brown (1.3% solution). No inhibition.

Fig. 4.

1. *Serratia* planted after 46 months exposure to 1: 180 acid fuchsin. No inhibition.
2. *B. anthracis* planted after 35 days exposure to 2% neutral red. No. inhibition.
3. *Staphylococcus aureus* planted after 24 hours exposure to 1.3% Grübler's Bismarck brown. No inhibition.

2,000 solution (Fig. 5, 2). Observations of this kind led to a study of the *degree* of resistance of *Serratia* to these dyes which are so toxic for certain members of the Gram-positive group.

In a preliminary experiment, enough aqueous gentian violet (Grübler) was added to a heavy suspension of a 24-hour old culture to make a 1:15,000 dilution. Plants were made from this tube onto agar at intervals, and the organisms were found to be still alive and vigorous at the end of 26½ months when the experiment was abandoned. Bacterial counts made during the early periods of this experiment showed that the bacteria were not growing; on the contrary their

numbers decreased. In the later periods, bacterial counts were not made on account of the danger of contamination.

The observations of Winslow and Brooke,[4] that organisms freed by washing from the protective action of proteins are more susceptible to toxic agents than unwashed organisms, led us to undertake experiments in which this point was borne in mind. With the colon organisms, our results seemed to confirm the findings of Winslow and

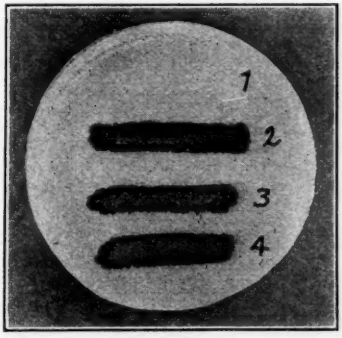

Fig. 5.

1. *Staphylococcus aureus* after exposure to gentian violet (1: 4500) for 45 minutes, at room temperature, and then three times centrifuged and washed. No growth.
2. *Serratia* after 4 hours exposure to 1: 2000 gentian violet, planted unwashed. No inhibition.
3. *Serratia* planted after 16 months exposure to 1: 4500 gentian violet. No inhibition.
4. *Serratia* planted after 46 months exposure to 1: 180 acid fuchsin. No inhibition.

Brooke; at least the organisms put up in gentian violet died sometime during the first ten days. In the case of *Serratia*, however, survival for many months was observed even when the organisms were washed free of media before being immersed in the dye.

With stronger dilutions of gentian violet the results were even more striking. Transplants onto agar made from specimens put up with a vaseline seal (to prevent drying) 16 months ago still show active growth tho the tubes contain 1 drop of 1% dye to 3 cc. of suspension (Fig. 6, 1). As the strength of dye is increased, a point is of course finally reached when the organism will not survive exposure. Yet even after 3 hours exposure to 1: 100 gentian violet *Serratia* grows actively in transplants.

[4]Winslow, C. E. A. and Brooke, Olive R. The viability of various species of bacteria in aqueous suspensions. J. Bact. 13, 235–243. 1927.

With acid fuchsin, the results are equally striking. Exposure to this dye at room temperature is almost without effect on this organism. Transplants onto agar made from a suspension of this organism put up 46 months ago show vigorous growth altho the tubes contain 4 drops of 2% acid fuchsin (National Aniline) to 2 cc. of suspension. (Fig. 5, 4).

It was thought that results of this kind might indicate the capacity of *Serratia marcescens* to assume some resistant form comparable to the spore. Tests, however of the thermal death points of young and old cultures showed no difference in behavior toward heat, evidence which hardly lends support to the hypothesis of a spore.

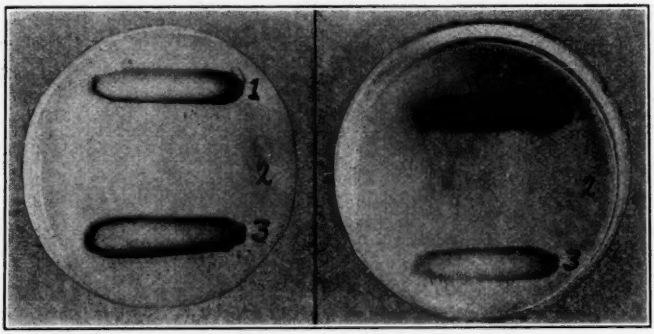

Fig. 6. Three streak inoculations of *Serratia* on one plate, showing effect of heat plus gentian violet.

1. After exposure at room temperature for 16 months to 1: 4500 dye solution. No inhibition.
2. After exposure for one hour to same dye solution but at 45°C. Complete inhibition.
3. After exposure to 45° C. for one hour without dye. No inhibition.

Fig. 7. Three streak inoculations of *Serratia* on one plate, showing effect of heat plus acid fuchsin.

1. After exposure at room temperature for 46 months to 1: 180 dye solution. No inhibition.
2. After exposure for one hour to same dye solution but at 45°C. Complete inhibition.
3. After exposure to 45°C. for one hour without dye. No inhibition.

Nor could any difference in filterability be demonstrated between the organisms at the beginning of the experiment and after long exposure to the dye, evidence which is not favorable to the hypothesis of a morphological change into a filter-passing form.

One point to which attention has been called in a previous communication[5] should be referred to again here, namely the striking effect in certain instances of a very slight rise in temperature upon

[5]Churchman, J. W. The effect of slight increase of temperature on the bacteriostatic power of gentian violet. J. H. H. Bull. 33, 227. 1922.

the action of dyes on bacteria. It was shown in the article referred to that an amount of gentian violet[6] quite without injurious effect when applied to the colon organism at room temperature even for long periods of time, might kill this organism when applied for 1 hour at 50° C. or even lower. It was also shown that this degree of heat alone would not kill the organism, nor would exposure to the dye, followed by heat.

The significance of the heat factor is strikingly shown if we compare the effect of a short exposure of *Serratia* to gentian violet plus heat, or acid fuchsin plus heat (45° C.) with a long exposure (many months) to the same dilutions without heat. Figs. 6 and 7 illustrate the results of such an experiment. In Fig. 7 three streak innoculations have been made on the plate. Streak No. 1 shows vigorous growth of this organism after standing in 1: 180 acid fuchsin at room temperature for 46 months. Streak No. 2 shows no growth at the line where the plate has been inoculated with the culture after exposure to 1: 180 acid fuchsin at 45° C. for 1 hour. Streak No. 3 shows the vigorous growth of the organism when subjected to the rise of temperature alone (45° C. for 1 hour), a growth which proves that this degree of heat, alone, will not kill this organism. Fig. 6 shows the results of a similar experiment with gentian violet.

[6]The experiments were at that time being done with Grübler's gentian violet.

NOTES ON TECHNIC

TECHNIC FOR THE ISOLATION OF EPITHELIUM. Procure the tissue, trachea, bladder, mouth, etc., from a freshly killed animal and place in physiological saline solution. Wash in this saline and then in an isotonic solution of sodium bicarbonate (1.5% solution) to remove any mucin which may be present. Then place in Muller's fluid dissociator.

Muller's Fluid	$K_2Cr_2O_7$....2.5 g. $NaSO_4$.....1 g. H_2O.....100 cc.	1 part
Physiological NaCl		9 parts

Allow the tissues to remain in this at room temperature for 36 hours. Remove the tissue from the fluid, take a small brush and lightly brush the surface of the epithelium. Transfer the cells to a microscopic slide and dry slightly. Fix in methyl alcohol, rinse in distilled water and stain with Harris' hematoxylin and eosin. Mount in balsam.

The same tissue may be used repeatedly until a microscopic examination shows a scarcity of epithelial cells, the tissue being placed back in the dissociating fluid when not in use. Care should be taken not to allow the specimen to stand too long a time without obtaining the material, as a too lengthy maceration loosens cells of the underlying tissue also.

The intestine is a little more difficult from which to prepare satisfactory slides, that is, slides free from debris; but by not allowing the tissue to remain in the macerating fluid more than 18 or 20 hours, this difficulty may be overcome.—M. LOIS CALHOUN, Vet. Anatomy Dept., Iowa State Col., Ames. Ia.

METHYLENE BLUE FOR STAINING THE DIPHTHERIA ORGANISM. For this purpose the medicinal grade of methylene blue is to be preferred. Avoid the products labelled "for bacilli". Two companies, one domestic, the other foreign, label certain grades of methylene blue with that designation. The domestic and the foreign products thus labelled are not apparently the same thing; and neither, in spite of the name, seems as good for staining the diphtheria organism as the medicinal grade. The latter can apparently be used safely for any of the purposes for which methylene blue is used by histologists or bacteriologists; there is therefore no need of keeping more than one grade in stock.

The Loeffler formula for methylene blue, containing a small amount of alkali is unnecessary for the purpose with modern medicinal methylene blue. In fact, use of the Loeffler formula is likely to cause excessive dealkylation, and result in too purplish a solution. A good formula for the purpose is a plain 1% aqueous solution of a methylene blue having about 90% dye content.—H. J. Conn

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

ANSELMIER, HÉLÈNE. Recherche sur la coloration rapid des cils des Ciliés. Pharmaceutica Acta Helvetiae, **5**, 33–41. 1930.

A review of various methods of staining protozoan cilia is given and the author offers the following procedure:

Fixation is of primary importance. Various fixatives may be used, but the author prefers 2 to 4% formalin employed by applying a drop or two on the live organisms.

Debris should be eliminated, and in heavy cultures this is attained by dilution. Chemical cleanliness of the slides is as important as when staining for bacterial flagella.

Casares-Gil's fluid is used for mordanting (70% alcohol, 30 cc.; tannin, 10 g.; hydrated aluminium chloride 18 g.; 10 g. zinc chloride in 10 cc. of water, added drop by drop; and rosanilin hydrochloride 1.5 g.); dilute 1: 4 and filter before using. The mordanting is sufficient when a variegated tinge appears on the edges, one minute usually.

After washing any of the following stains may be applied: Various dilutions of Giemsa's azure-eosin, Grübler (source of other stains not mentioned); acid fuchsin made up by Ziehl formula; saturated aqueous tropeolin; 10% aqueous nigrosin, with or without 5% phenol; methylene blue, with thymol, phenol, or formalin; Ziehl's basic fuchsin; Ehrlich's gentian violet; 1: 19 aqueous solution of collargol; 0.4% alcoholic, or 2% aqueous, safranin; 5% aqueous mercurochrome. In general stains used for bacteriological purposes may be applied for the rapid study of ciliates.

BARNHART, R. L. and EBERSON, F. A note on the microscopic diagnosis of acid-fast organisms. J. Lab. & Clin. Med., **16**, 421. 1931.

Gardner and Shunker find that a yellowish counterstain brings out the tubercle organism in bolder relief. The authors of this note have experimented with staining and counterstaining this group of bacteria, and find that equally satisfactory results are obtained by simply utilizing a yellowish light and dispensing with daylight or the daylight electric bulbs. Plain or frosted 50 to 75 watt bulbs are recommended and the usual Ziehl-Neelsen technic is used, without any counterstain.

Acid fast organisms from various sources appear a brilliant red, and their identification is simple and reliable if the light used has a yellowish tinge.

BOLCEK, L. Über die Zelloidineinbettung des Bouin-fixierten histologische Materials. Zts. Wis. Mikr. **47**, 334–5. 1930.

Material fixed in Bouin's fluid cuts poorly after celloidin embedding. The author supposes that picric acid forms potassium picrate with the potassium present in the tissues. As this salt is but slightly soluble in alcohol or ethyl alcohol, it greatly hinders celloidin penetration. A method in which alcoholic nitric acid is used for dissolving potassium picrate is proposed, as follows:

Wash Bouin fixed material in 70% alcohol, transfer to 80% for 10 to 24 hours, 96% for 24 to 48 hours, changing it 2 to 3 times. Next transfer to the following solution: cedar oil, 1 cc.; absolute alcohol, 8 cc.; origanum oil, 2 cc.; nitric acid conc., 1 cc. Treat in this for 10 to 24 hours for objects of about 10.5 cm. in diameter and 24 to 48 hours for larger ones. The material softens in this solution and is passed thru a few changes of 96% alcohol within 24 to 48 hours. Then proceed as usual for celloidin embedding.

COUCH, A. B. A method to soften tissue already embedded in paraffin. Science, **72**, 607–608. 1930.

The following treatment is supposed to soften plant material not especially tough when fresh, but which turns brittle after embedding. Pineapple leaves are handled as follows: Fix pieces not over 4 by 10 mm. in FAA (formula not given); dehydrate, clear in xylol and infiltrate as perfectly as possible. Block and trim so that the two edges and one end of the tissue will be directly exposed. (For longer pieces expose both ends.)

Store at about 30° C in 95% alcohol containing enough carbol fuchsin to make it pink. Well infiltrated material should not turn red thruout when transferred to water containing some carbol fuchsin.

Depending on the toughness of the tissue, softening may require 2 days to 3 weeks. Transfer to water for 2 to 24 hours before cutting. If the tissue becomes brittle towards the middle of the block repeat the water treatment.

Besides pineapple, the method has been tried on lily ovaries and it is supposed to be of general use for tissues which are not tough when fresh.

EARL, R. O. Rapid staining of plant tissues in the butyl alcohol procedure. Science, **72**, 562. 1930.

Total staining of various stem tissues following Zirkle's method (Science, **71**, 103, 1930; abs. in Stain Techn. **5**, 116, 1930) is recommended for its effectiveness and rapidity. Zirkle's procedure is followed until the last stage in butyl alcohol, to which safranin (no information given as to source or exact nature of this dye) has been added to saturation. Sections are left therein over night and then embedded. The second paraffin bath removes all color except from the specimens. Mounted sections are dipped in xylol, in a 1: 1 mixture of absolute alcohol and xylol, in xylol, and then counterstained with light green S. F., dissolved in clove oil. Xylol follows, then the xylol-alcohol mixture, and finally xylol again, from which the slides are mounted at leisure.

With buttercup roots it was found necessary to add a stage of absolute ethyl alcohol prior to counterstaining and to prolong the staining half an hour or longer.

HANNA, G. D. Hyrax, a new mounting medium for Diatoms. J. Royal Micr. Soc., **50**, 424–26. 1930.

Hyrax is recommended for mounting Diatoms as a medium superior to Canada balsam. It is a resin, a derivative of the well-known chemical naphthalene. The author, who discovered and realized the advantages of this medium, was not able to obtain a colorless substance. Messrs. Penn and Ruederich, (Box 26, Associated, California) succeeded in manufacturing a resin of so pale a color that slides mounted therewith were practically colorless. The refractive index of the hardened resin was found to be 1.82248.

Hyrax is soluble in benzene, xylene and several other organic solvents, not in alcohol or water. When hot it is extremely fluid and therefore displaces air from the objects being mounted very readily.

A few experiments proved that Hyrax does not alter some of the common stains used in biology. The author feels, however, that these few tests do not justify a general statement. Hyrax is neither acid nor basic, and this seems very desirable. Slides kept in a cabinet have not changed for a period of four years.

HEMENWAY, A. F. Some new methods and combinations in plant microtechnic. Science, **72**, 251–252. 1930.

Glycerin is a more satisfactory dehydrating agent than alcohol. It prevents plasmolysis, and permits better infiltration and cutting.

Leaves which have been killed in an aqueous fluid should be transferred to 10% aqueous glycerin in a flat dish and left until solution becomes as thick as pure glycerin. If the killing fluid used contained alcohol, transfer to glycerin of same percentage as the alcohol in the killing fluid. Wash in 95% ethyl alcohol, put thru 2 changes of absolute, and transfer to alcohol-xylol mixtures (3:1, 1:1, and 1:2 respectively) and finally to pure xylol. Embed as usual.

The following modification of the method for "dehydrating woody tissue for paraffin embedding," as given in Science for Jan. 24, 1930 has been used successfully. Transfer soft material, or if hard after treating it with HFl with 95% alcohol, (which according to Dr. Jeffrey is less injurious than water), to 10% glycerin. Transfer to equal parts of

glycerin and butyl alcohol after the glycerin has concentrated for 36 hours. Transfer to pure butyl alcohol for another 36 hours. Embed then as described in 1930. Material thus treated is less brittle and no harder than before dehydration. Prolonged paraffin immersion is harmless.

The author suggests that the addition of 8 to 10 parts of glycerin to preservatives such as alcohol-acetic-acid-formalin is efficacious in preventing rapid evaporation. (Important in dry climates).

The curling of woody sections may be avoided by flooding and leaving them on the blade for a few seconds with 1 part glycerin plus 1 part 95% alcohol and transferring them to the same fluid.

JENNISON, M. W. Detection of fungus mycelium in mildewed cotton fabrics. Science, **72**, 346–347. 1930.

The Pianese IIIb stain is recommended for the demonstrating fungus mycelia in mildewed cotton or cotton fabrics, as well as showing the extent of the infection. Wash the material in water or preferably in alcohol, stain 15 to 45 minutes in the Pianese stain (martius yellow, 0.01 gr; malachite green, 0.50 g.; acid fuchsin, 0.10 g.; H_2O, 150 cc; 95% ethyl alcohol, 50 cc.) Wash in water, decolorize in acid alcohol, dry and mount in Canada balsam or gum damar.

Cotton stains green, the fungus mycelium deep pink. The mycelium is naturally harder to demonstrate in dyed cloth than in raw cotton and undyed fabrics, but it can be detected usually in all cases. In the case of heavy fabrics it is recommended that one tear the fibers apart before mounting.

PETROFF, S. A. A useful cover-slip holder. J. Lab. & Clin. Med., **16**, 314–315. 1930.

The following cover-slip holder saves time and breakage in staining blood smears. Make a brass coil spring by winding 18-gauge spring wire around a 7 mm. round rod. Cut the coil so that each holder will consist approximately of 10 to 12 loops. Solder the coil spring between the ends of a cross wire spring (like the conventional cover-slip holder). Solder another piece of wire on one end of the coil spring leading from one end of the coil bent at right angles and running across, in a position to act as a guide in preventing the cover-slips from going too far in.

Prepare blood smears, mark and dry. Separate the loops of the coil by pressing the opposite sides, and insert the slips in the interspaces. Release the main outside wire, by which the spring loops will return to their normal position, clamping the cover-slips firmly. Place the loaded holder in a jar containing Wright's solution for one minute, then to equal parts of water and Wright's stain for two minutes and rinse in water. Remove excess water by touching the lower edges of the slips with blotting paper. Release the slips by pressing the two ends of the holder, blot, mount and examine.

POPOFF, N. La méthode des colorations en masse appliquée a l' histologie normal Bull. d' Histol. Appl. **7**, 300–302. 1930.

For a number of years embryos, embryonic organs and all material used for histologic demonstrations (University of Lausanne) have been stained *in toto*. The following combinations proved satisfactory: hemalum-eosin; hemalum-erythrosin-orange G; hemalum with Van Gieson; toluidin-blue-erythrosin; toluidin-blue-fuchsin; Weigert's resorcin fuchsin with eosin; same with Van Gieson; orcein-hemalum-erythrosin; orcein-hemalum-Van Gieson. (Sources of dyes not mentioned).

Any of the usual fixatives may be used, (formalin, formalin acetic, Bouin's, Gilson's or Zenker's). The following schedule is recommended for hemalum-eosin: After a more or less prolonged treatment in 80% alcohol, wash under tap water for at least 10 min., rinse in distilled water and transfer to hemalum (diluted with water 1:1) for 12 to 24 hours or more at 30 to 35° C. Place material on gauze rather than directly on the bottom of the staining jars. Subsequently wash under tap for at least 10 min., and rinse in distilled water.

Stain in 0.5% eosin or erythrosin in 60 to 70% alcohol for 5 to 6 hours or more without previous differentiation. Pass thru a series of alcohols (80% up to 100%), each except the final (*i. e.* 3rd) bath of absolute, containing a little of the counterstain, to avoid decolorization of the latter. Embed in paraffin as usual.

Staining 2 to 3 hours in orange G (1% in 80% alcohol) after the eosin, as above, gives still better results, especially for glands, skin, bone and cartilage.

SATO, KUNIO. Further study on the real nature of vital staining. Folia Anat. Jap. 8, 391–400. 1930.

Further experiments corroborate the author's idea, discussed in a previous paper, (Folia Anat. Jap., 8, 51–61, 1929) that vital staining is due to dye particles taken up by the cell body by a physical process.

If trypan blue is injected in the rabbit following KCl, more stain is observed in renal tubules than in a normal animal. If KCl follows trypan blue, very little is left. The reverse happens when $CaCl_2$ is used in the procedure. Trypan blue is made up of 2 constituents—a red highly diffusible substance, and a blue substance less diffusible. The color of excreted urine is blue when the stain is injected with KCl, (which loosens and swells the cells), and red when injected with $CaCl_2$, (because of the condensation of the cells). It seems of interest that granules observed in the renal tubules are violet-blue to blue in the first case and red-violet to violet in the second; that is they correspond to the color of excreted urine.

The renal cells loosened by potassium pass the stain easily, so that more comes out in the urine and much of the stain is deposited in the epithelium. Just the reverse is true for calcium. This indicates that storing of the stain in the living cell depends upon intracellular concentration of the stain molecules, which pass thru the cell body, and also upon the permeability of the cell.

SINTON, J. A. & MULLIGAN, H. W. The staining of malarial parasites in blood smears by the iron hematoxylin method. Ind. J. Med. Res. 17, 1329. 1930.

A method is given for removing hemoglobin and thus permitting malarial parasites in blood smears to be stained with iron hematoxylin.

Make smears and fix with osmic vapor (a procedure of minor importance). Immerse in Carnoy's fluid for 5 to 10 min. Wash in one change of absolute alcohol and leave a few minutes in absolute alcohol. Fixation for 15 to 30 minutes in Gilson's fluid, followed by 50% alcohol gives as good if not better results.

To remove malarial pigment transfer thru 95% alcohol to alkaline alcohol (95% alcohol, 96 parts; 1% caustic potash, 4 parts) for several hours. Pass thru graded alcohols to water Mordant in a 1% aqueous solution of Bordeaux red (source of dye not given) for 12 to 24 hours; this may be omitted. Mordant in 4% iron alum for 3 to 18 hours, preferably the latter, and rinse. Stain in Shortt's hematoxylin (hematoxylin, preferably Grübler's, dissolved by slowly boiling in 95 cc. of water, 1 g.; carbolic acid 5 cc.) Differentiate in 0.25 to 0.5% iron alum. Counterstain with eosin, pass thru graded alcohols, xylol and mount in balsam or euparal.

For a rapid technic Dobel's hematein is suggested, but it is decidedly poorer than the longer procedures. For this procedure fix in either Carnoy's or Gilson's fluids, rinse in 70% alcohol. Mordant for at least 10 min. in 1% iron alum; rinse in 70% alcohol. Stain in 1% hematein in 70% alcohol, rinse in 70% alcohol. Differentiate in the iron alum; pass thru the alcohols, dehydrate and mount.

SKUPIENSKI, F. X.[1] Sur la coloration vitale de *Didinium nigripes* (FR). Acata Societatis Botanicorum Poloniae. No. 3, 204213, 1929. Warsaw.

Neutral red, methylene blue were used mainly, also to some extent Janus green, dahlia, Bismarck brown and methyl violet, for the vital staining of a Myxomycete, *Didinium nigripes*. Macro-cultures were grown in test tubes and in Ehrlenmeyer flasks on agar with decoctions of carrot, potato or hay. Micro-cultures were grown on slides with carrot and potato bouillon. Stains were added in solution in the proportion of 1: 5000 to both media. Checks proved that neutral red, methylene blue, and Janus green do not alter nor retard the usual developmental cycle of this Myxomycete. Micro-cultures offered the possibility of cytologic studies of vitally stained organisms.

When neutral red is added to agar, it appears pink and the condensation water still deeper pink. Spores incubated upon this medium germinate within a few hours in darkness and at 15° C. As *Didinium* develops, the color of the medium and of the condensation drops becomes paler and finally decolorizes completely. Small slightly pink-colored plasmodia appear on the surface within a few days. These fuse into one large body of the same color. After a few more days the body develops progressively on the upper portion of the substratum and becomes a deeper pink than previously, even more so than the water of the condensation drops.

[1]This paper is abstracted in greater detail than usual because the original is not readily available to American readers.

The plasmodium breaks up into more or less regular fragments united by protoplasmic filaments. As these fragments become more individualized, their color becomes more intense, and they become spheroid. These bodies elongate gradually and their upper larger portions become the heads, while the lower portion develops into the stalks of the sporangia. Morphological changes are accompanied by color changes. The spheroid plasmodium is intensely pink, but during its elongation the color disappears from the lower portion and becomes intensified in the upper. When the sporangium attains its final shape, it shows a blood red head and a milk white stalk. This red is soon covered up by the calcareous crust which develops on mature sporangia. Mature sporangia are therefore similar, whether grown on a medium with stain or without.

Studying various stages of the micro-cultures under the microscope, 3 kinds of vacuoles were observed: a) elementary vacuoles, which appear in the spore and persist in the zoospore, myxamoebic and plasmodial stages. These are stained pink and show red inclusions similar to vacuoles of higher plants; b) pulsating vacuoles, which refuse to stain at all; c) digestive vacuoles, stained most intensely and with red particles which they digest. Some of the so-called elementary vacuoles refuse to stain, and they reduce osmic acid to some extent.

Besides vacuoles, numerous corpuscles staining a deep red were observed in the cytoplasm. These may be the metachromatic corpuscles of Dangeard, the chemical nature of which Guillermond described in many of his papers.

The nuclei of zoospores and myxamoebae do not stain. Both lose motility and become round prior to division. Digestive and pulsating vacuoles are absent and the only elementary vacuoles present stain more intensely. The staining of digestive vacuoles is of great interest. In the central part of a plasmodium are enormous vacuoles, each enclosing an ungerminated spore or a cyst, or a number of bacteria. A whole cyst within the vacuole, including the nucleus, is stained, and one may observe the rapidity with which such a cyst is killed. Undigested particles are rejected.

The plasmodium chooses a relatively dry location before forming sporangia. This stage requires special handling. Fragments of the stained plasmodium are transferred with a spatula to a drop of distilled water on a slide and covered with a cover glass.

Microscopic observations reveal that the protoplasm is very condensed at this stage and contains a number of spherical granules stained red. These granules swell under the influence of water, and become pink-colored vacuoles.

In the condensed protoplasm of the early sporangium, prior to the appearance of spores, numerous spheroid red-colored corpuscles are found. These also swell up in water and become colored vacuoles, a number of crystals appear at this stage at the periphery of the sporangium. Normal nuclei are unstained, degenerative ones show a red coloration. Early spores are large and have quite thin membranes. It is surprising to observe a large spheroid vacuole, or rarely two, in a young spore which is colored pink and contains a large crystalline dark red body. The body diminishes in size as the spore matures, but still exists in the ripe spore. When these are inoculated and zoospores develop, they show completely unstained vacuoles. The crystalline body is absent. The author suggests that the body may be some reserve substance used up in cellular metabolism.

Methylene blue (1: 5000), stains the membranes and achromatic portion of the nucleus, especially the nuclear membranes of zoospores, myxamaebae and plasmodia. In the sporangium the stalk alone is colored. When neutral red and methylene blue are mixed, the organisms are differentially stained. The sporangial head shows red, while the stalk is blue.

Janus green stains only the achromatic portions of the nucleus. Bacteria present in digestive vacuoles stain intensely. Janus green, which is considered a chondriosomal stain, failed to show these bodies in all the stages of the fungal cycle.

SMITH, H. M. Golgi staining. Turtox News, (*Published by General Biol. Supl. House*), **8**, 91–92. 1930.

A simplified modification of the Golgi method is proposed. Fix brain in 10% formalin for an indefinite period of time and cut slices about 2 μ thick exactly perpendicular to the surface of the desired portion. Transfer to 4% potassium bichromate for 3 to 5 days. Wash in distilled water, blot, and place in 0.75% silver nitrate. Agitate frequently for the first 15 minutes. Change the silver nitrate solution and let stand 24 or more hours. Rub off precipitate from the surface after a few hours. Absolute darkness is not essential, but bright light must be avoided.

After the silver impregnation, rinse the tissue in distilled water and place into 83% alcohol. Change the alcohol several times within 3 to 4 hours to eliminate all traces of free silver nitrate. Transfer for one hour each thru 95% alcohol, absolute alcohol and ether alcohol. Transfer to a thick celloidin solution and incubate for 1 to 2 hours. Block and immerse in chloroform for several hours; transfer to 95% alcohol for over night. Prolongation of this treatment may cause decomposition of the stain.

Cut sections 60 to 80 μ thick and transfer to absolute alcohol with 10% chloroform. Transfer to xylol; choose best sections, and mount in benzol-damar without cover glasses.

The cerebrum is well impregnated if pyramidal cells are seen with their axons and dendrites, the cerebellum if Purkinje cells show up in their entirety. The method has been used successfully on human autopsy material (adult as well as young), and on monkey brain.

STOUGHTON, R. H. Thionin and orange G for the differential staining of bacteria and fungi in plant tissues. Ann. Appl. Biol., **17**, 162–164. 1930.

A method is proposed for staining various plant parasites in host tissue, including *Bacterium malvaecerum*, the cause of the angular leaf spot in cotton, where slime production interferes with staining.

Pass paraffin sections thru xylol and alcohols to water. Stain 1 hour (or only 5 min., if free-hand sections are used) in 0.190 thionin in 5% phenol. (Thionin used was that of the British Drug Houses and the "Soloid" tabloids of Messrs Burroughs, Wellcome & Co.; but it is mentioned that apparently any good brand would be satisfactory.) Pass thru graded alcohols up to absolute. Differentiate 30 sec. to 1 min. in a saturated solution of orange G (source not named) in 100% alcohol until the sections become yellowish green. Wash in absolute, pass thru xylol-alcohol, xylol and mount in balsam.

A modification which may prove of value in certain cases is to remove the orange G by running the sections down to water and back to xylol. All the orange G will disappear and the parasites will seem very conspicuous against unstained walls.

The parasite is stained violet-purple, with deep purple nuclei, cellulose walls yellow or green, lignified tissue blue, tissue nuclei pale blue with purple nucleoli and chromosomes deep blue on a purple spindle.

YOSHIDA, Y. "Kollodionharz Patent." Ein neues Einbettungsmittel. Folia Anat. Jap, **9**, 13–16. 1930.

A new embedding medium is proposed instead of celloidin. (It is a product registered as No. 42799 in the Japanese patent office.) Collodium Ph. J. and commercial pine resin are the ingredients used.

The collodium is filtered and concentrated by evaporation to about half its volume. The resin is broken up, heated at low temperature upon paper to absorb the easily melted impurities. It is dried, pulverized and hot water is added to it with constant stirring. The balsam forms a lump while the water gets yellow. This lump is removed and pulverized, and two volumes of absolute alcohol are added. Dissolving takes about 24 hours. Then filter the solution, after further alcohol has been added. Evaporate this filtrate on a sand bath. Repeat this procedure about four times. One eventually obtains an amorphous yellow brownish substance devoid of the usual odors of resin and which seems more viscid when melted than the original product.

Add 15% balsam to the collodium and use as in the celloidin method. Sections of desired thickness are cut and, unlike celloidin sections, these may be kept indefinitely in 0.5% phenol. Before staining, pass the sections thru alcohol and ether-alcohol. Use alcohol vapor to soften old pieces, as ether-alcohol is too strong for this material.